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DEVELOPMENT OF *CALCEOLARIA* CHROMOPLASTS IN THE PRESENCE OF HERBICIDES AFFECTING CAROTENOID BIOSYNTHESIS

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Three herbicides (SAN 9789, SAN 9785 and amitrole) were used to study the biosynthesis of carotenoids and the connected structural changes in chromoplasts of *Calceolaria* flowers. All three herbicides affected the biosynthesis of total carotenoids, especially of lutein. The strongest inhibition was obtained by SAN 9789, which considerably inhibited the production of lutein and the normal formation of »chromoplast internal membranes« (CIMs). Amitrole moderately influenced both the development of lutein and CIMs. SAN 9785 did not cause any conspicuous fine structural modifications in the chromoplasts, although it reduced the content of pigments significantly. The results are discussed with regard to the known action of these herbicides on the biosynthesis of carotenoids.

Introduction

In the chromoplasts of *Calceolaria* flowers the pigment-containing structures consist of numerous, in the stroma concentrically arranged, membranes, which are usually called »chromoplast internal membranes« (CIMs; Liedvogel et al. 1976). These membranes develop by ingrowth from the inner membrane of the plastid envelope (Wrischer and Ljubešić 1984). Their formation begins already in young chloroplasts

Dedicated to Prof. Zvonimir Devidé on the occasion of his 70th birthday.

in flower buds. During the growth of the flowers the number of CIMs increases constantly, while the thylakoids slowly disappear. The main pigment in these chromoplasts is lutein. It has been stated that in membranous chromoplasts pigments are located mainly in the CIMs (Liedvogel et al. 1976).

The relation between the formation of chromoplast structures and the biosynthesis of carotenoids is still not understood well enough. A useful tool for this study are herbicides that impair carotenoid biosynthesis. We tested the effect of three of these herbicides on the formation of CIMs and on the biosynthesis of pigments in the *Calceolaria* chromoplasts. All three herbicides are known to affect, directly or indirectly, the carotenoid biosynthesis in chloroplasts. The »bleaching« herbicides SAN 9789* and amitrole inhibit at different points the biosynthesis of ß-carotene (Buschmann and Grumbach 1982, Laskay et al. 1983, Barry and Pallett 1990), while SAN 9785 affects carotenoids only indirectly by changing the desaturation reactions of some fatty acids of plastid lipids (Laskay et al. 1983, Leech et al. 1985).

Material and Methods

Sublethal concentrations of herbicides were applied in all experiments. Flower buds (3—5 mm in diameter) of *Calceolaria herbeohybrida* Voss. were immersed for 24 hrs in a tap water solution of one of the following herbicides: SAN 9789 (0.2 and 0.02 mM), amitrole (1 mM) and SAN 9785 (0.2 mM). Control buds were immersed for the same time in pure tap water.

Small pieces of petal tissue were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.2 at 1°C), washed in the same buffer, and post-fixed in 1% OsO_4. The dehydrated tissue was embedded in araldite and the thin sections stained with uranyl acetate and lead citrate. Thin sections were examined in an Opton (Oberkochen) EM-10 A electron microscope.

The pigments were extracted in 80% acetone and the carotenoids were separated by thin-layer chromatography on silica gel G plates with the mixture of petrol ether (60—80°C): ethyl acetate : diethylamine (58 : 30 : 12) as solvent and measured spectrophotometrically (Specol 10 — Zeiss, Jena) at 450 nm (Davies 1976).

Results

The colour of control, i. e. of untreated *Calceolaria* flowers changed from pale-green (in 3 — 5 mm large buds) to yellow (in fully developed 32 — 35 mm large flowers). None of the herbicides used significantly affected the growth of the flowers. After the treatment with SAN 9789 and amitrole the flowers became pale-yellow or even white, while the treatment with SAN 9785 did not cause any change of the colour in the flowers, which became normally yellow.

* Abbreviations: SAN 9789, 4-chloro-5-(methylamino)-2-(*a,a,a*-trifluoro-*m*--tolyl)-3(2H)-pyridazinone; SAN 9785, 4-chloro-5-(dimethylamino)-2-phenyl 3-(2H)-pyridazinone; amitrole, 3-amino-1,2,4-triazole

Untreated flower buds contained young chloroplasts with small grana consisting of 2 — 3 thylakoids (Fig. 1). The formation of CIMs, by the ingrowing of the plastid envelope, was observed in flowers having 10 mm in diameter. In fully grown flowers (32—35 mm large) the irregularly shaped chromoplasts were filled with numerous concentrically arranged CIMs, while thylakoids were absent. There were also saclike membranous structures connected with CIMs. Some small plastoglobules were present in a very empty stroma (Fig. 2).

SAN 9789 strongly affected the formation of CIMs. In light-green fully grown flowers, which developed after the treatment with the lower concentration (0.02 mM) of this herbicide, the CIMs were present, but had an undulated appearance (Fig. 4). After the treatment with a 0.2 mM solution of SAN 9789 they were even more disorganized and formed a dense network (Fig. 5).

Amitrole also affected the development of CIMs. In young flowers these membranes had still a normal appearance, but in fully grown ones they were often tightly stuck together. Large plastoglobules, with electron-translucent centers, appeared in the stroma of these chromoplasts (Fig. 3).

SAN 9785 did not considerably influence the development of the *Calceolaria* chromoplasts, except that a somewhat reduced number of CIMs was found in some of them.

None of the herbicides caused any fine structural changes in other cell constituents, except in chromoplasts.

Young chloroplasts in flower buds contained small quantities of chlorophyll (Wrischer and Ljubešić 1984), which completely disappeared during the chromoplast differentiation. In fully grown flowers the main pigment was lutein (92.4%), while the rest was ß-carotene (4.9%) and an unidentified carotene (2.7%).

All herbicides applied lowered the content of pigments of the flowers, although with various intensity. In flowers pretreated with amitrole the content of total carotenoids reached 55% of that of the control, in SAN 9785 pretreated flowers 39%, and in SAN 9789 pretreated ones only 28% (Table 1). This inhibition concerned primarily the content of lutein. The strongest inhibition was caused by a 0.2 mM solution of SAN 9789 (19% of that in the control), and the weakest by amitrole (38% of the control). The contents of ß-carotene and of the unidentified carotene also varied and were usually higher than in untreated flowers. An especially high accumulation of the unidentified carotene was found in flowers pretreated with SAN 9789 (Table 1).

Table 1. Content of carotenoids (mg/g fr. wt.) of untreated and treated fully grown *Calceolaria* flowers.

	total carotenoids	lutein	β-carotene	unidentified carotene
control	0.153	0.142	0.008	0.004
SAN 9789	0.043	0.027	0.007	0.009
SAN 9785	0.059	0.044	0.010	0.005
amitrole	0.084	0.054	0.016	0.013

Discussion

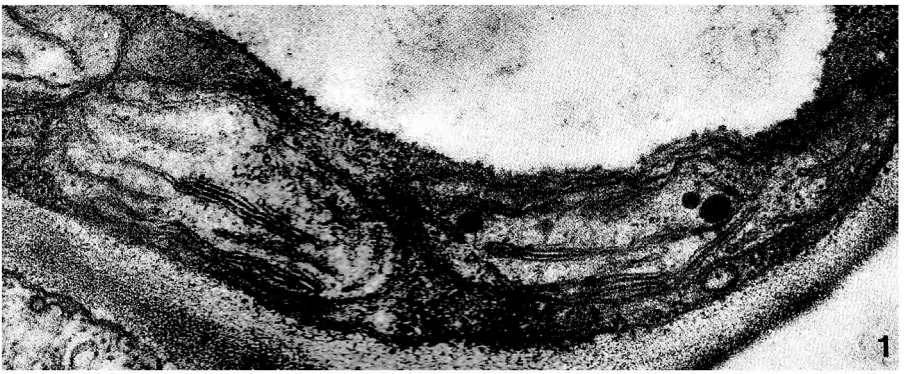
The data reported in this paper show that the three herbicides used have various effects on the fine structure and the biosynthesis of pigments in *Calceolaria* chromoplasts. The strongest inhibition is caused by SAN 9789. The content of carotenoids is reduced to about a third of that in untreated flowers, and CIMs, although detectable in the chromoplasts, are structurally rather disorganized. Pigments are obviously necessary for the stability of these membranes. Amitrole has a somewhat weaker effect on the chromoplasts. However, the sticking of the CIMs and the presence of large plastoglobules in the chromoplast stroma indicates an accumulation of lipids, which probably originate from degraded membranes. It is surprising that SAN 9785 has almost no effect on the fine structure of *Calceolaria* chromoplasts, although it noticeably drops the pigment content in the flowers. It is supposed that SAN 9785 affects the carotenoid biosynthesis only indirectly (Leech et al. 1985). Slight changes in the lipid content, i. e. in the content of some fatty acids (Laskay et al. 1983), could possibly reduce the normal integration of pigments into the CIMs. SAN 9785 produced also only minor changes in the fine structure of chromoplasts of daffodil flowers (Hloušek-Radojčić and Ljubešić 1988), and of chloroplasts of barley leaves (Laskay et al. 1983, Leech et al. 1985).

The relatively high amounts of ß-carotene and of an unidentified carotene, which accumulate in *Calceolaria* flowers after the treatment with amitrole and SAN 9789, are contrary to the results reported for chloroplasts and some chromoplasts. It has been repeatedly stated that the »bleaching« herbicides affect primarily carotenes (Bartels et al. 1967, Bartels and McCullough 1972, Buschmann and Grumbach 1982, Hloušek and Ljubešić 1985, Hloušek-Radojčić and Ljubešić 1988, Sagar and Briggs 1990). The weak inhibition of carotenes in *Calceolaria* is perhaps the consequence of a specific feature in carotenoid biosynthesis of normal *Calceolaria* flowers, because the main pigment and the only xanthophyll is lutein (Wrischer and Ljubešić 1984). It is supposed that the biosynthetic pathway of lutein is different from those of ß-carotene and other xanthophylls (Barry and Pallett 1990). In *Calceolaria* this lutein biosynthetic pathway is possibly particularly sensitive to the »bleaching« herbicides.

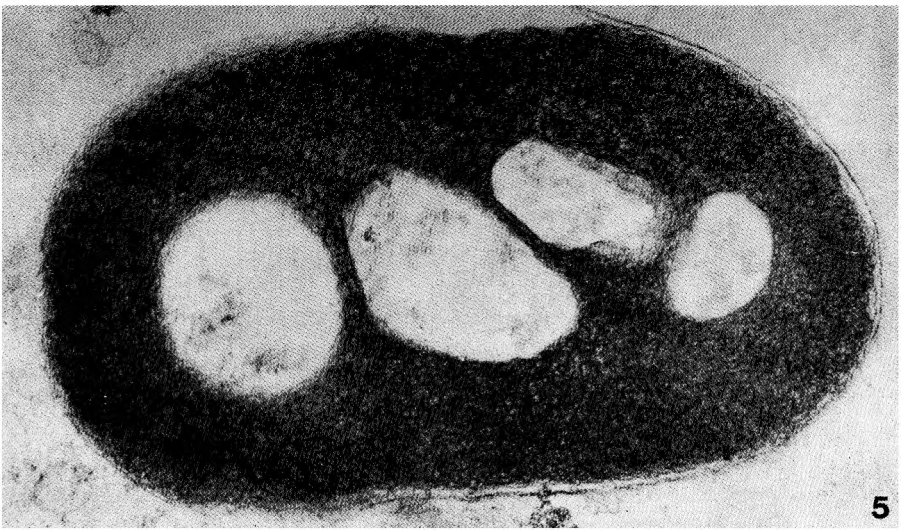
Fig. 1. Chloroplasts from a young, untreated flower (5 mm in diameter). 44,000 : 1.

Fig. 2. Chromoplast from a normal fully grown flower (32 mm in diameter). 35,000 : 1.

Fig. 3. Chromoplasts from a pale-yellow fully grown flower pretreated with amitrole (1 mM) with stuck CIMs (arrow) and large plastoglobules. 21,000 : 1.



Figs. 1—3.



Figs. 4—5.

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Fig. 4. Chromoplast from a pale-yellow fully grown flower pretreated with SAN 9789 (0.02 mM) with undulated CIMs, 43,000 : 1.

Fig. 5. Chromoplast from a pale-yellow fully grown flower pretreated with SAN 9789 (0.2 mM) with highly damaged CIMs. 45,000 : 1.

SAŽETAK

RAZVOJ KROMOPLASTA U CVIJETU KALCEOLARIJE U PRISUTNOSTI HERBICIDA KOJI INHIBIRAJU BIOSINTEZU KAROTENOIDA

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Autori su studirali utjecaj herbicida SAN 9789, SAN 9785 i amitrola na biosintezu karotenoida i ultrastrukturu kromoplasta u cvijetu kalceolarije. Sva tri herbicida inhibiraju biosintezu karotenoida, napose luteina. Najjača inhibicija postiže se primjenom herbicida SAN 9789, koji znatno smanjuje količinu luteina i normalnu izgradnju membrana kromoplasta. Amitrol nešto blaže utječe na biosintezu, kako luteina, tako i membrana. SAN 9785, međutim, ne uzrokuje uočljivije ultrastrukturne promjene u kromoplastima, iako je količina pigmenata u cvjetovima znatno smanjena. Autori su u raspravi razmotrili značenje dobivenih rezultata s obzirom na djelovanje pojedinih herbicida na biosintezu karotenoida.

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